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14. ABSTRACT Chronic pain is among the most prevalent health problems in the United States today, affecting 10% of the population and costing the U.S. billions of dollars each year in health care expenses, lost income, and lost productivity. Genetic differences among individuals in pain response physiology are partially responsible for observed variation in chronic pain development and maintenance. To identify genes affecting inter-individual variability in chronic pain response we are using a state of the art reference population of laboratory mice (Diversity Outbred mice). Diversity Outbred (DO) mice are a unique population of laboratory mice designed to maximize allelic variation throughout the genome (Churchill, Gatti et al 2012). Each DO mouse is genetically unique. Unlike fully inbred strains, cohorts of DO mice approximate the levels of genetic (allelic) diversity found in human populations. The levels of segregating phenotypic and allelic diversity in DO mice allow for high precision for mapping regions of the genome that condition complex traits. Identifying genes whose allelic variants condition susceptibility to chronic pain development will further our understanding of the molecular mechanisms underlying chronic pain. This information, in turn, promises to facilitate improved methods for individualized chronic pain treatment and prevention.					
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Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	7
Reportable Outcomes.....	7
Conclusion.....	7
References.....	7
Appendices.....	8

INTRODUCTION

The goal of this study was to identify genes affecting inter-individual variability in chronic pain nociception using a state of the art population of laboratory mice (Diversity Outbred mice) designed for precision mapping of complex traits. The study design involved

- 1) using a formalin assay of nociception to assess susceptibility to chronic pain in a cohort of 300 Diversity Outbred (DO) mice,
- 2) genotyping of the DO mice using the 77K SNP Mouse Universal Genotyping Array (MEGA MUGA),
- 3) performing quantitative trait locus (QTL) mapping to identify regions of the genome that condition chronic pain response, and
- 4) assessing candidate genes for chronic pain identified by QTL mapping using existing bioinformatics resources.

BODY

The Statement of Work for W81XWH-11-1-0762 outlined tasks associated with two technical objectives:

- ***Technical Objective 1: Phenotype and perform genetic mapping analysis in a genetically diverse reference population of mice.***
- ***Technical Objective 2: Identify chronic pain susceptibility candidate genes and pathways.***

A Gantt chart showing the tasks and timelines is shown in Appendix 1, Figure 1. A summary of accomplishments for each of the tasks associated with the technical objectives is provided below.

Technical Objective 1: Phenotype and perform genetic mapping analysis in a genetically diverse reference population of mice. We phenotyped 300 Diversity Outbred (DO) mice (150 males; 150 females) and males and females (eight mice of each sex) of the eight inbred founder lines of the DO mice using the formalin assay of nociception. Each of the 300 DO mice were genotyped using the GeenSeek 77K SNP “MEGA MUGA” genotyping array that is specifically designed to assay genetic diversity in DO mice. The formalin response phenotype data and genotype data were analyzed using a Hidden Markov Model (HMM) statistical algorithm to identify regions of the mouse genome that contributed to chronic pain response.

Task 1.1: Establish a phenotyping test environment

Completed. The phenotyping apparatus used in this study consisted of a 16-chamber plexiglass enclosure. Each chamber was monitored by an individual video camera to record animal behavior following exposure to formalin.

Task 1.2: Obtain Diversity Outbred Mice

Completed. 300 Diversity Outbred mice (150 males and 150 females) were obtained from The Jackson Laboratory. Mice were obtained at 4 weeks of age and held until 13-17 weeks of age at which time they were phenotyped. Due to aggressive behavior, males were singly housed. Female mice were housed in cohorts of 5 mice per cage.

Task 1.3: Obtain DO founder lines

Completed. Eight male and eight female mice of each of the 8 inbred strains (A/J, C57BL/6J, 129S1Sv/ImJ, NOD/ShiLtJ, NZO/H1LtJ, CAST/EiJ, PWK/PhJ, and WSB/EiJ) that were used to establish the Diversity Outbred mouse population were phenotyped using the same protocols as for the DO mice (see Task 1.5).

Task 1.4: Phenotyping refresher training

Completed. Prior to undertaking the phenotyping for this study, all personnel were trained according to a single Standard Operating Procedure (SOP) (described in IACUC protocol 01011/USAMRMC protocol 09141008) under the supervision of JAX veterinary science staff to ensure consistency in the phenotyping and compliance with all animal welfare guidelines.

Task 1.5: Phenotyping of DO cohort mice

Completed. The formalin test of nociception is a well-characterized model of chemically induced, post-tissue injury tonic pain. The phenotyping methods were described in IACUC protocol 01011 (“Cell cycle control”)/USAMRMC protocol 09141008. Subcutaneous formalin injection produces an easily identifiable, reproducible behavioral response in mice that consists of licking, biting, and flinching of the affected paw. Behavioral response to formalin occurs in two phases, with a total duration of approximately 1 hour in rodents. All 300 DO mice were successfully phenotyped using the formalin test of nociception. Mice were transported to the experimental room and left undisturbed in the glass behavior chamber to habituate for 1 hour under ambient light before beginning the experiment. Twenty microliters of 2.5% formalin solution were injected subcutaneously into the plantar surface of the right hind paw using a 50 microliter syringe with a 30-gauge needle. Injected mice were individually placed into the behavior chambers, located on a flat, glass surface to allow clear monitoring of the injected paw by individual video camera.

The videotaped formalin injection responses for each animal were processed (scored) manually. The time each animal spent licking or biting the formalin injected paw in 5-min intervals up to 60 min beginning immediately after formalin injection. The amount of time mice spent exhibiting licking or biting behavior between 10 and 60 minutes was used to measure pain response induced by nociceptor sensitization. All mice were euthanized immediately following the completion of the formalin phenotyping protocol.

Task 1.6: Phenotyping of DO founder lines

Completed. The phenotyping of the eight inbred DO founder lines was performed as described above for Task 1.5.

Task 1.7: Genotype DO Mice and Progenitors

Completed. Each DO mouse in this study was genotyped using a specially designed genotyping array (Mouse Universal Genotyping Array; MUGA). The MUGA array measures ~77,000 SNPs and was specifically designed for optimal genotyping of DO mice. Progenitor genotype data were obtained from the SNP repository hosted by the Center for Genome Dynamics at The Jackson Laboratory.

Task 1.8: Perform QTL Mapping

Completed. QTL analysis identified two genotype/phenotype association peaks associated with formalin response: one peak on chromosome 8 contained the known protein coding pain-related gene cadherin 8 (*Cdh8*); the second peak on chromosome 13 contained the transcription factor activator of basal transcription 1 (*Abt1*) (Figure 2). According to the Mouse Genome Informatics knowledgebase, *Cdh8* is known to play a role in thermal nociception, but this is the first study to suggest a role for *Cdh8* in chronic pain response. *Abt1* is a novel candidate pain

gene, but its functional and gene expression data support its putative role in pain response (see results for Task 2.1, below).

The allelic effect plots shown in Figure 3 illustrate the phenotypic effects of each DO founder strain's genetic contribution at each position along the Chr 8 (A) and Chr 13 (B) QTL. For the chromosome 8 QTL, the allelic contribution from A/J (yellow) and WSB (purple) is reported to condition high sensitivity to formalin-induced tonic pain, while allelic contributions from CAST/EiJ (green) and PWK/PhJ (red) are reported to condition low sensitivity to formalin-induced tonic pain. For the chromosome 13 QTL, the allelic contribution from CAST/EiJ (green) and 129S1/SvImJ (pink) is reported to condition high sensitivity to tonic pain, while WSB/EiJ (purple) is reported to condition low sensitivity to tonic pain.

Technical Objective 2: Identify chronic pain susceptibility candidate genes and pathways.

We used haplotype association mapping (HAM) analysis with high-density SNP data to identify chronic pain susceptibility candidate genes within the QTL regions defined in Objective 1. We used publicly available computational tools and bioinformatics resources to identify biological pathways involving these candidate genes.

Task 2.1: Assess relevance of candidate genes identified in Task 1.8

Completed. The QTL mapping (Task 1.8) identified a single gene on chromosome 8, *Cdh8*. Although not previously associated with chronic pain, the developmental and tissue specific gene expression and functional information associated with this gene in the Mouse Genome Informatics database suggest the gene is likely to be a novel chronic pain gene. The *Cdh8* gene encodes a protein that is a type II cadherin from the cadherin superfamily. It is an integral membrane protein that mediates calcium-dependent cell-cell adhesion. *Cdh8* is expressed in a subset of neurons in the dorsal horn (DH) of the spinal cord, as well as by a small number of neurons in the dorsal root ganglia (DRGs) (Suzuki et al. 2007). Functionally, *Cdh8* is putatively involved in synaptic adhesion, axon outgrowth and guidance. Previous research by Suzuki et al. (2007) demonstrated that *Cdh8* is involved in “coupling between cold-sensitive sensory neurons and their dorsal horn targets.”

Genetic mapping also identified a 10 Mb QTL on chromosome 13 containing the candidate gene *Abt1* (activator of basal transcription 1). Studies in mouse suggest that the protein encoded by *Abt1* likely activates basal transcription from class II promoters by interaction with TATA-binding protein and the class II promoter DNA (de Planell-Saguer et al. 2009). Like *Cdh8*, *Abt1* has not previously been associated with chronic pain, but its functional information and gene expression patterns support its putative role in pain response. *Abt1* is expressed throughout the brain, spinal cord, and dorsal root ganglion and is known to be involved in spinal cord motor neuron differentiation (MGI, 2013). *Abt1*'s reported interaction with acetaminophen, a common anti-inflammatory and analgesic compound, further suggests a role for *Abt1* in pain response (Baker et al. 2012).

Task 2.2: Pathway analysis

Completed. See description of results for Task 2.1.

Task 2.3: Prepare and submit manuscript for publication

In progress. The manuscript that reports the methods and results of the chronic pain discovery project is in preparation for submission to a peer-reviewed scientific journal.

KEY RESEARCH ACCOMPLISHMENTS

The key research accomplishments for the project include the following:

- First use of the Diversity Outbred mice to identify genes associated with chronic pain.
- Identification of two loci containing the candidate genes *Cdh8* and *Abt1*.
- The first reported association of *Cdh8* and *Abt1* as plausible candidates for chronic pain susceptibility.

REPORTABLE OUTCOMES

Manuscript in preparation.

CONCLUSION

The research reported in this report describes the first application of DO mice to genetic and bioinformatic approaches for the discovery of new genes related to chronic pain nociception. Genetic linkage mapping in DO mice produced a much more precise and efficient mapping result than conventional mapping populations. The ability to map a 2 Mbp QTL is a marked improvement over conventional two-strain mapping cross studies, which typically result in pain-related confidence intervals of ~30 Mbp. Precise genetic mapping combined with integrative genomic techniques allowed us to identify two QTL, each with a single candidate pain gene (*Cdh8* and *Abt1*), in a single two year study – an endeavor that typically requires multiple mapping and fine-mapping studies over a period of several years.

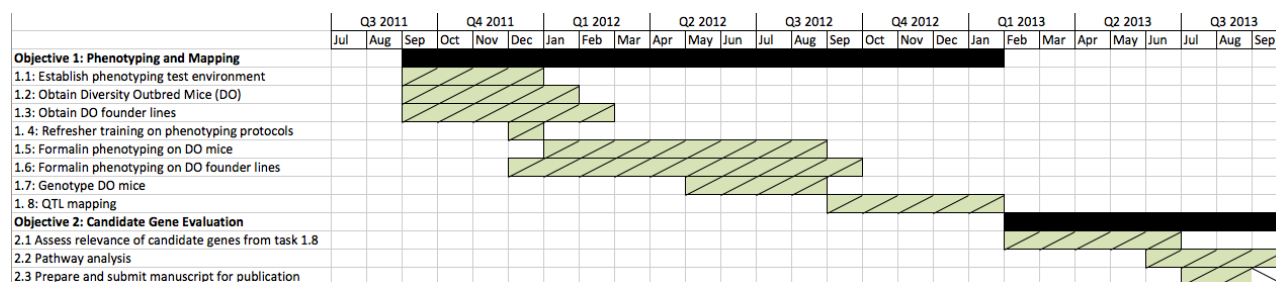
We have demonstrated here that genetic mapping in the DO mapping population is a comparatively fast, cost-effective, high-precision approach to the identification of novel pain-related candidate genes. Combining the precise mapping afforded by the DO with integrative genomics dramatically improves our ability to find the genetic basis of complex traits, including those related to chronic pain response. The information generated by this research promises to facilitate the long term goal of developing improved methods of individualized chronic pain prevention and treatment in veterans as well as the general population.

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APPENDIX 1 - SUPPORTING DATA

Figure 1. Gantt chart showing progress towards completion of tasks associated with Objectives 1 and 2 of the Systems Genetics of Chronic Pain project (W81XWH-11-1-0762).



Gantt chart key:

Black line = Expected timeframe for objective

Shaded with forward slash = Completed

Unshaded with backwards slash = Task in progress

Figure 2. Genome-Wide plot of genetic linkage mapping results for formalin response in a group of 300 DO Mice. The most significant QTL peaks are located on chromosomes 8 and 13.

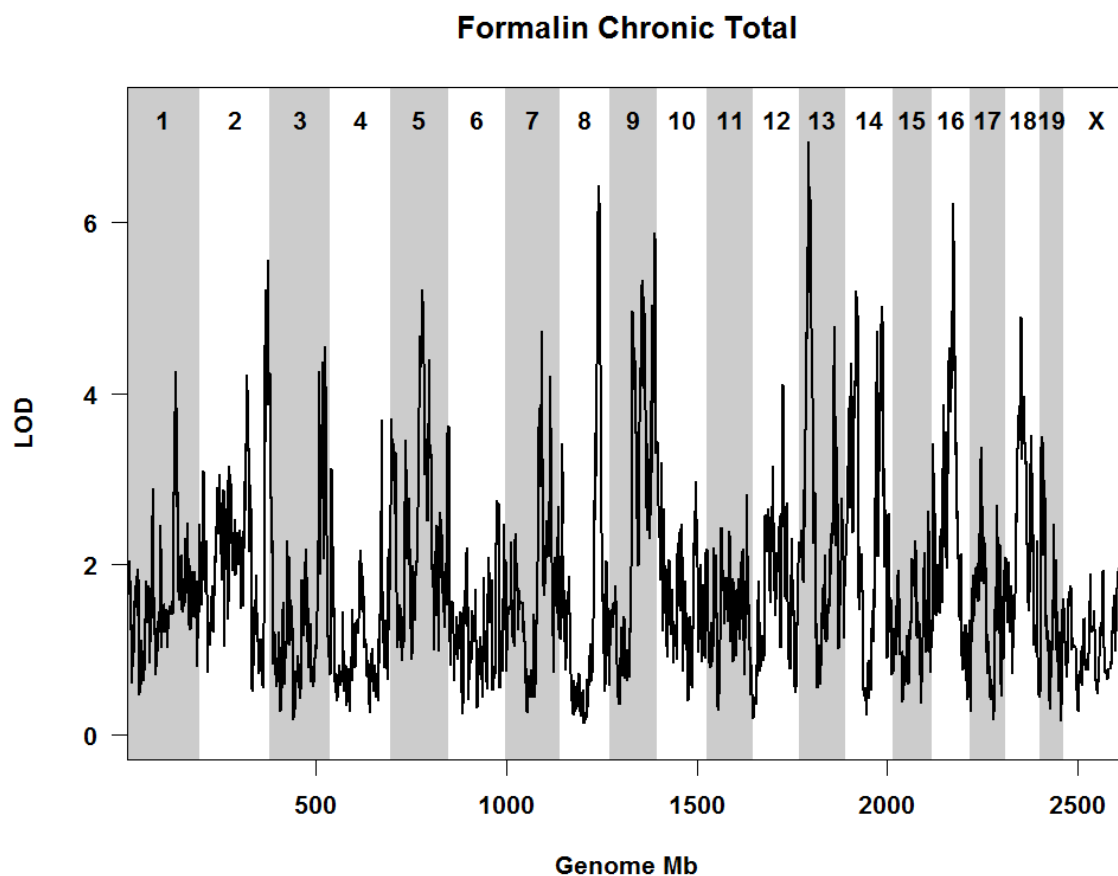


Figure 3. Allelic effect plot for the DO formalin genetic linkage mapping results on Chr 8 (A) and Chr 13 (B). Colored lines represent the phenotypic effect of each DO founder strain's allelic contribution at each SNP locus across the chromosome. Within the Chr 8 QTL, the allelic contribution from A/J (yellow) and WSB (purple) is reported to condition high sensitivity to formalin-induced tonic pain, while allelic contributions from CAST/EiJ (green) and PWK/PhJ (red) are reported to condition low sensitivity to formalin-induced tonic pain. Within the Chr 13 QTL, the allelic contribution from CAST/EiJ (green) and 129S1/SvImJ (pink) is reported to condition high sensitivity to tonic pain, while WSB/EiJ (purple) is reported to condition low sensitivity to tonic pain.

